

# Recovery Comparison of Two Virus Concentration Methods from Wastewater Using Cell Culture and Real-Time PCR

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Received: 5 March 2012 / Accepted: 4 June 2012 / Published online: 6 July 2012  
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**Abstract** Enteric viruses are shed in the feces and may be present in environmental waters. Their detection in wastewater, even at low concentration, is a major challenge. In this study, recoveries of *Echovirus 7* (EV7), virions and RNA in wastewater, using virus concentration methods were determined to evaluate the detection of infectious viruses and the possibility of recovering viral genomes. Two virus concentration methods, PEG precipitation method and two-phase separation method, were applied to recovery experiments of EV7–virions from wastewater, in parallel with recovery experiments of EV7 RNA. The titration of EV7 virions was carried out by cell culture using human rhabdomyosarcoma tumor tissue and the EV7 RNA quantification was performed by real-time PCR. The mean recovery yields of EV7 virions using the PEG precipitation method and the two-phase separation method were  $78.5 \pm 10.99$  and  $83.1 \pm 0.28$  %, respectively. Besides, EV7 RNA recoveries obtained using the PEG precipitation method were four times higher than

those using the two-phase separation method. According to our results, the two methods enable to concentrate both infectious viruses and viral genomes. Moreover, considering the protocol time and cost together with the ratio of the EV7 virion recovery to the EV7 RNA recovery, the two-phase separation method (83.1/2.71 %, or 30.6) seems to be more appropriate for selective concentration of viral virions than the PEG precipitation method (78.5/10.33 %, or 7.6).

## Introduction

Viruses are a major cause of water-related diseases [4]. They are constantly found in raw sewage of large populations and in environmental waters affected by fecal contamination, sometimes leading to waterborne outbreaks [28]. According to public health data, the most important enteric viruses are the *enteroviruses* (EV), *adenoviruses* (HAdV), *noroviruses*, *rotaviruses*, *astroviruses* as well as *hepatitis A* (HAV) and *E* viruses. More than 100 virus types can colonize the gastrointestinal tracts and cause a wide variety of illnesses, inducing variable epidemiological features and some water-related diseases [36].

*Enteric viruses* are excreted in the feces of infected individuals in high concentrations and transmitted mainly by the fecal–oral route via contaminated water [30]. Indeed, they have been detected in various water samples [8, 9, 13, 19, 26, 33]. The detection of viruses in wastewater, even at low concentrations, is a major challenge. Because usually only a few viral particles are present in wastewater samples, it is necessary to concentrate the viruses from a large volume of water, commonly from 13 ml to 2 l [13, 23]. Hence, many virus concentration methods have been developed: the two-phase separation

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[11], the polyethylene glycol (PEG) precipitation [20, 21], the ultracentrifugation [15, 34], and methods using an electropositive filter [16] and an electronegative filter [10]. Furthermore, two virus concentration methods from wastewater are advised by WHO for environmental poliovirus surveillance [37]. The first one uses bags with sorbent macroporous glass to trap viruses and the second uses the two-phase separation, which is applied in this study. Regarding the comparison of virus concentration methods from water, several studies have been conducted using quantitative polymerase chain reaction (qPCR) [1, 7, 31]. Until now, to our knowledge, there is only one published study in which the recovery of naked viral genomes from wastewater, using two virus concentration methods, have been evaluated [10].

During the virus replication in the host cell, many proteins and viral genomes are produced from the mRNA separately and assembled into the infectious virions at the end of this step. A large number of viral genomes are released into the feces of patients and are discarded in wastewater. PCR amplification detects genomes of both infectious and non-infectious viruses even though the infectious virus ratio in water remains unknown in the absence of cell culture. Thus, it is important to know the recovery of both infectious and non-infectious viruses in the sample for the concentration method used.

The aim of this study was to compare two virus concentration methods, the PEG precipitation and the two-phase separation, to evaluate the recovery of infectious viruses and the possibility to recover viral genomes of *Echovirus 7* (EV7) that were artificially seeded in wastewater. The titer of EV7 was quantified by cell culture and the EV7 RNA was quantitatively determined by qRT-PCR. Wastewater was spiked with EV7 virions and RNA, to provide information about which method is more suitable, taking into consideration time and cost.

## Materials and Methods

### Spiking Experiments and Wastewater Samples

Raw wastewater samples used for spiking experiments were collected from a wastewater treatment plant (WWTP), located at 70 km from Casablanca in Morocco. One single pool of 26 l was collected in plastic bottles. Eighteen liters were used for EV7 virions experiments and 8 l for EV7 RNA. In order to damage viruses and nucleic acids which could be present in wastewater, samples were first autoclaved at 121 °C for 20 min. Then, they were divided into 52 volumes of 500 ml and stored at +4 °C until use.

For each virus concentration method, two EV7 virions concentrations 1.00E+06 TCID<sub>50</sub>/100 µl and 1.00E+03

TCID<sub>50</sub>/100 µl (tissue culture infectious dose 50 %) were seeded in nine replicates in 500 ml of autoclaved wastewater. The EV7 RNA was also seeded in four replicates at two final RNA concentrations: 1.00E+04 and 1.00E+02 RT-PCR Unit (RT-PCR U). In all experiments, negative control without virus or RNA spiking was included. Each spiked sample was vigorously shaken before concentration proceeding. The titer of EV7 virions stock used in spiking experiments was 1.00E+08 TCID<sub>50</sub>/100 µl and the concentration of EV7 RNA stock used was 1.00E+06 RT-PCR U. The titers were calculated as follows: Virions volume seeded in wastewater = (Concentration of seeded virions × 500 ml)/1.00E+08 TCID<sub>50</sub>/100 µl and RNA volume seeded in wastewater = (Concentration of seeded RNA × 500 ml)/1.00E+06 RT-PCR U.

### Concentration Methods

The seeded wastewater samples were concentrated using two methods. The first one “PEG precipitation” was carried out as described elsewhere [20] with modifications. Five hundred milliliters of sample were clarified by centrifugation (30 min, 1,000×g) and the pellet was resuspended in 10 ml of the supernatant. The remaining portion of supernatant was saved. Chloroform was added to the resuspended sample to a concentration of 10 % (v/v, Sigma, USA) and mixed, and the mixture was centrifuged again (5 min, 1,000×g). The first and second supernatants were combined. The volume was measured and the pH was adjusted to 7.5. The PEG–NaCl technique as previously described [22] was used with modification. The combined supernatants were supplemented with NaCl and PEG until a final concentration of 2.2 % (w) NaCl (Sigma, St Louis, USA) and 7 % (w) PEG 6000 (Fluka, Steinheim, Germany) was obtained. The mixture was stirred 4 h at 4 °C and then centrifuged for 2 h at 2,000×g at 4 °C. Supernatant was discarded and the pellet was suspended in 5 ml of phosphate buffer saline (PBS).

In the second method “two-phase separation” [37], 500 ml of sample was centrifuged for 10 min at 1,000×g. The pellet was stored and the pH of the supernatant was adjusted to neutral (pH 7–7.5). Usually only a few milliliters of NaOH 1 N is needed. The supernatant was mixed with 39.5 ml of 22 % dextran (Sigma Aldrich, St Louis, USA), 287 ml of 29 % PEG 6000 (Fluka, Steinheim, Germany), and 35 ml of 5 N NaCl (Sigma, St Louis, USA) and kept in constant agitation for 1 h at 4 °C using a magnetic stirrer. After overnight incubation at 4 °C in a separation funnel, the entire lower layer and the interphase were collected. The pellet from the first centrifugation was resuspended in this concentrate and the suspension was extracted with 20 % volume of chloroform by shaking vigorously, followed by centrifugation for 5 min at

1,000×g. The supernatant (10 ml) was recovered and decontaminated by antibiotic (e.g., penicillin G and streptomycin to final concentrations of 100 IU/ml and 100 µg/ml, respectively).

#### Titration of EV7 Virions and Samples

EV7 strain used in this study was obtained from patient fecal samples and maintained in our laboratory. The human rhabdomyosarcoma tumor tissue (RD) cell lines were cultivated in minimum essential medium (MEM, Gibco) with 2 % fetal bovine serum (FBS), penicillin and streptomycin, and were incubated at 37 °C with 5 % CO<sub>2</sub> for 5 days.

To determine the viral titer of EV7, the TCID<sub>50</sub> values were calculated by the Reed and Muench equation [7, 27]. The titer of EV7 stock used (1.00E+08 TCID<sub>50</sub>/100 µl) was determined using serial tenfold dilutions in a 96-well plate. Cells were plated at a density of 2 × 10<sup>4</sup> cells/ml in MEM with 10 % (v/v) FBS. After 24 h of incubation at 37 °C with 5 % of CO<sub>2</sub>, 100 µl of tenfold serial dilutions of virus were added per well with 8 replicates for each dilution. The plates with MEM with 2 % (v/v) FBS were incubated for 7 days at 37 °C with 5 % CO<sub>2</sub>. Virus replication was monitored daily under an inverted microscope. Before the titration with the same protocol, wastewater concentrates were filtered through filters with 0.22 µm pore size (Millipore, Billerica, USA). Negative and positive controls were included in each plate.

#### RNA Extraction

The High Pure Viral Nucleic Acid Kit (Roche, California, USA) was used to extract the RNA from the samples according to manufacturer's instructions. RNA extracts obtained were performed on 200 µl of sample concentrate and eluted in 50 µl.

#### Quantification of EV7 RNA

The quantitative reverse transcription-PCR (qRT-PCR) was performed using Superscript III Platinum One-Step Quantitative RT-PCR System (Invitrogen, California, USA) with an ABI PRISM 7500 Fast Real-Time PCR System (Applied Biosystems, California, USA). In brief, five microliters out of 50 µl of the extracted RNA were mixed with 20 µl of a reaction mixture containing 12.5 µl of 2× buffer, 4 mM of MgSO<sub>4</sub>, 50 nM of ROX Reference Dye, 0.5 µl of Superscript III RT/Platinum Taq Mix, 0.4 µM of forward primer (5'-CCCTGAATGCGGCTAA TCC-3'), 0.4 µM of reverse primer (5-ATTGTCACCATA AGCAGCCA-3'), and 0.2 µl of 10 µM TaqMan probe (5'-FAM-AACCGACTACTTTGGGTGTCCGTGTTTC-T AMRA-3') [25]. Subsequently, the mixture was added to

96-well plate (Applied Biosystems, California, USA) and was incubated at 50 °C for 30 min and 95 °C for 5 min followed by 45 cycles at 95 °C for 15 s and at 60 °C for 1 min.

In order to draw the standard curve, EV7 RNA was diluted by serial tenfold dilution. The samples and the standard samples were applied to RT-PCR at the same time followed by analysis using the SDS software (Version 2.0.3, Applied Biosystems), to obtain quantitative data on EV7 RNA RT-PCR U in the well. One RT-PCR U is defined as the concentration of the last positive dilution with Ct value less than 40 cycles. Three wells were used for each sample, negative and positive controls were included in each run. The average was used for the subsequent calculation.

#### Inhibition Test

To assess the potential presence of PCR inhibitors, the RNA of *mengovirus* strain MC<sub>0</sub> (ATCC VR-1957) (kindly provided by Albert Bosch, Department of Microbiology, University of Barcelona, Barcelona, Spain) was used as an internal control (IC). In each EV7 RNA spiking experiment, a 1.00E+03 RT-PCR U of *mengovirus* RNA was tenfold diluted in each RNA sample directly after nucleic acid extraction (sample with IC), and compared with positive control which includes tenfold diluted *mengovirus* RNA in RNase-free water. Negative control was also included.

The quantification of *mengovirus* was carried out through a real-time procedure following the same conditions as for EV7 RNA with the reverse primer Mengo209 (5'-GAAG TAACATATAGACAGACGCACAC-3'), the forward primer Mengo110 (5'-GCGGGTCCTGCCGAAAGT-3'), and the FAM-MGB probe Mengo147 (5'-ATCACATTACTGG CCGAAGC-3') [24].

#### Statistical Analysis

Percent recoveries of EV7 virions and RNA were calculated using the following equation: percent recovery = the number of recovered viruses / the number of seeded viruses × 100.

The Mann-Whitney U (MWU) test was used for EV7 virions tests, to examine mean recoveries differences from nine trials. The MWU test is a nonparametric version of the independent samples and does not require a normal distribution of the data. The independent variable is the percent recovery. All statistical analyses were carried out with GraphPad Prism software version 5.03 (GraphPad Software, [www.graphpad.com](http://www.graphpad.com)). Differences were considered significant if *P* was < 0.05.

**Results**

**Inhibition Test**

Data obtained from the EV7 RNA experiments demonstrated that all samples with IC were found positive for the presence of *mengovirus* RNA. The comparison between the Ct value mean obtained by the PEG precipitation method and that obtained for positive control showed  $30.42 \pm 0.37 \%$  (Ct value mean  $\pm$  SD), while a mean Ct value of  $30.62 \pm 0.11 \%$  was obtained for the two-phase separation method. That explains the absence of PCR inhibition in all RNA quantification tests.

**EV7 Virions/RNA Recoveries**

The yields of both the virus concentration methods were determined by spiking EV7 virions and RNA in wastewater using cell culture and qRT-PCR. Results showed that the two methods were able to concentrate both seeded infectious EV7 virions and RNA, as reported in Table 1. The EV7 virions were recovered at a relatively high level,  $78.5 \pm 10.99 \%$  (Ct mean  $\pm$  standard deviation) for the PEG precipitation and  $83.1 \pm 0.28 \%$  for the two-phase separation method. The statistical analysis of EV7 virions indicates that spiked wastewater with  $1.00E+06$  TCID<sub>50</sub>/100  $\mu$ l showed a mean recovery of  $70.72 \pm 19.37 \%$  for the PEG precipitation, whereas the two-phase separation showed  $83.3 \pm 11.23 \%$ . However, the difference of the average was not statistically significant ( $n = 9, P = 0.195$ , MWU test).

Similar results were obtained for spiked wastewater with  $1.00E+03$  TCID<sub>50</sub>/100  $\mu$ l of EV7 virions. The mean recovery was  $86.27 \pm 9.88 \%$  for the PEG precipitation, while the two-phase separation showed a mean of  $82.9 \pm 8.48 \%$ . However, the difference of the average was not statistically significant ( $n = 9, P = 0.453$ , MWU test).

Recoveries of EV7 RNA obtained for the PEG precipitation and the two-phase separation were  $10.33 \pm 2.85$  and  $2.71 \pm 1.95 \%$ , respectively. The mean recovery of EV7 RNA, resulting from four repeated experiments was four times higher for the PEG precipitation than that obtained for the two-phase separation method.

**Discussion**

*Enteroviruses* are recommended as a virologic marker for water quality in different studies, because they are environmental resistant, abundant, and easily detected by molecular methods and are of great importance in public health. In the WHO guidelines for environmental surveillance, the *poliovirus* was used as a model to determine the recovery of two-phase separation concentration method. In this study, EV7 which is an *enterovirus* was chosen as a model to represent *enteric viruses* because in our previous study, when the PEG precipitation method was used, no EV7 have been detected in wastewater samples [2]. We suggest that perhaps the PEG precipitation did not concentrate EV7.

PCR problems are commonly related to the presence of enzyme inhibitors which are also concentrated with viral particles [2, 5]. The absence of PCR inhibition in this study showed that the autoclaving may also remove inhibitors from wastewater samples.

The difference between the two methods to concentrate the seeded EV7 virions was not significant, which explains that the two methods were able to concentrate virions similarly for both  $1.00E+06$  TCID<sub>50</sub>/100  $\mu$ l and  $1.00E+03$  TCID<sub>50</sub>/100  $\mu$ l concentrations. For the RNA experiments, the mean recovery obtained using the PEG precipitation was four times higher than that obtained using the two-phase separation method. That may result from the centrifugation time of 2 h used in the PEG precipitation protocol which increased the RNA precipitation. The detection of virions

**Table 1** Mean recovery yields of EV7 virions/RNA from wastewater by PEG precipitation and two-phase separation methods

Inoculum	No. of TCID <sub>50</sub> or RT-PCR U <sup>b</sup> of EV7		No. of trials	% Recovery of trials (mean $\pm$ SD)	% Recovery (mean $\pm$ SD)
	Inoculated	Recovered			
PEG precipitation					
Virion	$1.00E+06$	$6.15E+05$	9	$70.72 \pm 19.37$	$78.5 \pm 10.99$
	$1.00E+03$	$8.63E+02$	9	$86.27 \pm 9.88$	
RNA	$1.00E+04$	$1.24E+03$	4	$12.35 \pm 3.4$	$10.33 \pm 2.85$
	$1.00E+02$	$8.32E+00$	4	$8.31 \pm 3.86$	
Two-phase separation					
Virion	$1.00E+06$	$8.33E+05$	9	$83.3 \pm 11.23$	$83.1 \pm 0.28$
	$1.00E+03$	$8.29E+02$	9	$82.9 \pm 8.48$	
RNA	$1.00E+04$	$1.33E+02$	4	$1.33 \pm 0.95$	$2.71 \pm 1.95$
	$1.00E+02$	$4.10E+00$	4	$4.1 \pm 1.02$	

<sup>a</sup> Tissue culture infectious dose 50 %

<sup>b</sup> RT-PCR Unit

with higher recoveries than those obtained for RNA is in agreement with a previous study [10], reporting the yields of spiked *Poliovirus 1* (PV1) virions and RNA, ( $50 \pm 8.9 \%$ ) and ( $3.4 \pm 1.6 \%$ ) using Mg-method, ( $22 \pm 9.3 \%$ ) and ( $5.2 \pm 4.9 \%$ ) using Al-method, respectively.

We made some modifications in the PEG precipitation protocol. The elution of pellet was performed using PBS instead of lysis buffer, to be able to amplify the viruses by cell culture after the concentration step. The time of stirring was also reduced to 4 h to decrease the concentration time. Comparing the time and cost of the protocols, the PEG precipitation requires less than 8 h while the two-phase separation requires two half days with overnight incubation. The latter protocol needs more reagent than the PEG precipitation, such as dextran, and twice the quantity of PEG and NaCl, which increases the cost of the method.

Traditionally, the PEG has been used as a virus precipitating agent in water in several studies [14, 17, 21, 32] but it can also precipitate DNA and RNA [3, 18, 35]. Therefore, the ability of PEG to precipitate RNA may provide an increase in the recovery for PCR (e.g., when detecting *noroviruses* from foods). The purpose of using the EV7 RNA experiments was to project the state of genome in wastewater which could become naked by treatment or transport. In this study, the free RNA was tested to demonstrate that both the virus concentration methods can concentrate RNA, even if the presence of viral genome has not been reported as a causative agent of disease. We suggest that if RNA is not recovered as efficiently as virions by a virus concentration method, the method is expected to have selectivity in detecting virions, although this hypothesis depends on the ratio of infectious virions and RNA (defective virions), as published previously [10].

Although, the presence of viral genome has not been reported as a causative agent of disease. However, the recovery of viral genome should be determined for the virus concentration method used. In previous studies, viruses have been detected from water using cell culture, PCR, or qPCR. Most of them were optimized by spiking different infectious viruses such as: HAV and HAdV [30], HAV [7], *Influenza A virus* [6], PV1 [29], and *Echovirus 1* and *Coxsackievirus B5* [12]. The cell culture is useful to detect infectious viruses. Moreover, wastewater concentrate may also be analyzed by molecular biology techniques (frequently PCR or qPCR). The use of PCR is relatively rapid and specific and may detect a small quantity of genome but PCR fails to discern between infectious and non-infectious particles which can be defective virions or naked viral genomes.

In conclusion, our study revealed that the two methods were able to concentrate EV7 virions similarly. The PEG precipitation method resulted in a better recovery of RNA

when compared to the two-phase separation. Moreover, considering the protocol time and cost together with the ratio of the EV7 virion recovery to the EV7 RNA recovery, the two-phase separation method ( $83.1/2.71 \%$ , or 30.6) seems to be more appropriate for selective concentration of virions than the PEG precipitation method ( $78.5/10.33 \%$ , or 7.6). However, a logical follow-up would be to study the performance of both the methods using wastewater sampling in quest for some *enteric viruses* to assess the most relevant method for all enteric viruses.

**Acknowledgments** The authors thank Prof Albert Bosch from the University of Barcelona (Barcelona, Spain) for providing us with the internal control.

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